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| (54) Title: FERMENTATIVE PREPARATION PROCESS FOR CYTOSTATICS AND CRYSTAL FORMS THEREOF  |  |  |  |
| (57) Abstract<br><br>The invention relates to a process for concentrating epothilones in culture media, a process for the production of epothilones, a process for separating epothilones A and B and a strain obtained by mutagenesis for the production of epothilones, as well as aspects related thereto. Crystal forms of epothilone B are also described.   |  |  |  |
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## FERMENTATIVE PREPARATION PROCESS FOR CYTOSTATICS AND CRYSTAL FORMS THEREOF

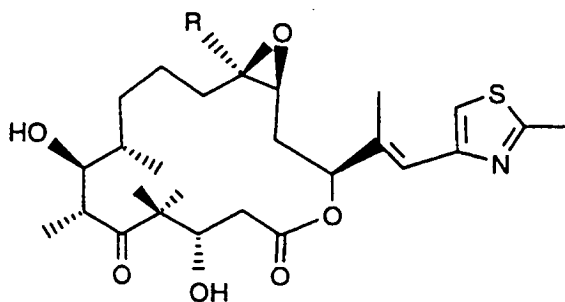
The invention relates to a new biotechnological preparation process that can be used on an industrial scale for the production of epothilones, especially a process for concentrating these compounds in the culture medium, as well as a new strain for the fermentative preparation of these compounds. The invention also relates to new crystal forms of epothilones, especially epothilone B, obtainable by the new processes, their usage in the production of pharmaceutical preparations, new pharmaceutical preparations comprising these new crystal forms and/or the use of these compounds in the treatment of proliferative diseases such as tumours, or in the production of pharmaceutical formulations which are suitable for this treatment.

Background to the invention:

Of the existing cytotoxic active ingredients for treating tumours, Taxol® (Paclitaxel; Bristol-Myers Squibb), a microtubuli-stabilising agent, plays an important role and has remarkable commercial success. However, Taxol has a number of disadvantages. In particular, its very poor solubility in water is a problem. It therefore became necessary to administer Taxol® in a formulation with Cremophor EL® (polyoxyethylated castor oil; BASF, Ludwigshafen, Germany). Cremophor EL® has severe side effects; for example it causes allergies which in at least one case have led even to the death of a patient.

Although the Taxan class of microtubuli-stabilising anti-cancer agents has been commended as "perhaps the most important addition to the pharmaceutical armoury against cancer in the last decade" (see Rowinsky E.K., Ann. rev. Med. 48, 353-374 (1997)), and despite the commercial success of Taxol®, these compounds still do not appear to represent a really great breakthrough in the chemotherapy of cancer. Treatment with Taxol® is linked with a series of significant side effects, and a few primary classes of compact tumours, namely colon and prostate tumours, respond to this compound only to a small extent (see Rowinsky E.K., *inter alia*). In addition, the efficacy of Taxol can be impaired and even completely neutralised by acquired resistance mechanisms, especially those based on the overexpression of phosphoproteins, which act as efflux pumps for active ingredients, such as "Multidrug Resistance" due to overexpression of the multidrug transport glycoprotein "P-glycoprotein".

Epothilones A and B represent a new class of microtubuli-stabilising cytotoxic active ingredients (see Gerth, K. et al., J. Antibiot. 49, 560-3 (1966)) of the formulae:



wherein R signifies hydrogen (epothilone A) or methyl (epothilone B).

These compounds have the following advantages over Taxol®:

- a) They have better water-solubility and are thus more easily accessible for formulations.
- b) It has been reported that, in cell culture experiments, they are also active against the proliferation of cells, which, owing to the activity of the P-glycoprotein efflux pump making them "multidrug resistant", show resistance to treatment with other chemotherapy agents including Taxol® (see Bolag, D. M., et al., "Epothilones, a new class of microtubule-stabilizing agents with a Taxol-like mechanism of action", Cancer Research 55, 2325-33 (1995)). And
- c) it could be shown that they are still very effective *in vitro* against a Taxol®-resistant ovarian carcinoma cell line with modified  $\beta$ -tubulin (see Kowalski, R. J., et al., J. Biol. Chem. 272(4), 2534-2541 (1997)).

Pharmaceutical application of the epothilones, for example for tumour treatment, is possible in an analogous manner to that described for Taxol, see for example US 5.641.803; US 5.496.804; US 5.565.478).

In order to be able to use the epothilones on a larger scale for pharmaceutical purposes, however, it is necessary to obtain appropriate amounts of these compounds.

Until now, the extraction of natural substances by means of myxobacteria, especially the epothilones from the cell strain *Sorangium Cellulosum Soce90* (deposited under no. 6773 at the German Collection of Microorganisms, see WO 93/10121) was described in literature. In order to obtain a satisfactory concentration of the natural substances, especially the epothilones, in the culture medium for the subsequent extraction, previously an adsorbate resin based on polystyrene was always added, for example Amberlite XAD-1180 (Rohm & Haas, Frankfurt, Germany).

However, the disadvantage of this process is that, on a large scale, it leads to an abundance of problems. Valves are impaired by the globules of resin, pipes can block, and apparatus may be subject to greater wear due to mechanical friction. The globules of resin are porous and therefore have a large inner surface area (about 825 m<sup>2</sup>/gram resin). Sterilisation becomes a problem, as air enclosed in the resin is not autoclaved. Thus, the process cannot be practicably carried out on a large scale using resin addition.

On the other hand, without adding resin globules, a satisfactory concentration of epothilones cannot be achieved in the culture medium.

Surprisingly, the requirements for finding a way out of this dilemma have now been found, enabling a satisfactory concentration of natural substances to be obtained from microorganisms, in particular myxobacteria, which produce epothilones such as epothilone A or B, in particular a concentration of epothilones A and B, in the culture medium, without the addition of resins, and thus enabling production of these compounds, especially epothilones to be carried out on a technical and industrial scale without the above-mentioned disadvantages.

#### Detailed description of the invention

One aspect of the invention relates to a process for concentrating epothilones, especially epothilone A and/or B, in particular epothilone B, in a culture medium, in order to produce these compounds on a biotechnological scale, the process comprising microorganisms which produce these compounds, especially myxobacteria (as producers of natural

substances), whereby a complex-forming component which is soluble in the culture medium is added to the medium.

A further aspect relates to the corresponding culture medium, which comprises a corresponding complex-forming component and microorganisms, especially myxobacteria, in particular of the genus *Sorangium*, which are suitable for producing epothilones, especially epothilone A and/or B.

A further aspect of the invention relates to a process for the production of epothilones, especially epothilone A and/or B, especially the two pure compounds, in particular epothilone B, which is characterised in that the epothilones are obtained by working up a culture medium for the biotechnological preparation of these compounds, which comprises as producers of natural substances microorganisms, especially myxobacteria, that produce these compounds, and to which a complex-forming component that is soluble in the culture medium is added, and the subsequent purification and, if desired, separation of the epothilones, for example epothilone A and B.

A fourth aspect of the invention relates to a method of separating epothilones, especially epothilones A and B from one another, which is characterised by chromatography on a reversed-phase column with an eluant comprising a lower alkyl cyanide.

A further aspect of the invention relates to a strain of *Sorangium cellulosum* obtained by mutagenesis, which under otherwise identical conditions, produces more epothilones than *Sorangium cellulosum* Soce90.

A further aspect also relates to new crystal forms of epothilone B.

The general terms used hereinabove and hereinbelow preferably have the meanings given hereinbelow:

Where reference is made hereinabove and hereinbelow to documents, these are incorporated insofar as is necessary.

The prefix "lower" always indicates that the correspondingly named radical contains preferably up to a maximum of 7 carbon atoms, in particular up to 4 carbon atoms, and is branched or unbranched. Lower alkyl may be for example unbranched or branched once or more, and is e.g. methyl, ethyl, propyl such as isopropyl or n-propyl, butyl such as isobutyl, sec.-butyl, tert.-butyl or n-butyl, or also pentyl such as amyl or n-pentyl.

A culture medium for the biotechnological preparation of epothilones which contains microorganisms that produce these compounds, especially myxobacteria, as producers of natural substances, is primarily a medium which comprises a corresponding (naturally occurring or also obtainable by cultivation or in particular by genetic modification) microorganism, especially a myxobacterial strain which is in a position to produce these compounds, in particular a myxobacterium of the genus *Sorangium*, preferably (in particular for epothilone production) a microorganism of the type *Sorangium Cellulosum Soce90* (see WO 93/10121), or a biomaterial derived therefrom (for example by mutagenesis or recombinant gene technology) or from parts of this myxobacterium, especially a correspondingly derived strain, in particular the strain having the reference BCE33/10 or mutants thereof, and in addition, together with water, preferably other conventional and appropriate constituents of culture media, such as biopolymers, sugar, amino acids, salts, nucleic acids, vitamins, antibiotics, semiochemicals, growth media, extracts from biomaterials such as yeast or other cell extracts, soy meal, starch such as potato starch and/or trace elements, for example iron ions in complex-bound form, or suitable combinations of all or some of these constituents and/or also analogous additions. The corresponding culture media are known to the person skilled in the art or may be produced by known processes (see e.g. the culture media in the examples of the present disclosure, or in WO 93/10121).

One preferred myxobacterium is a strain selected by UV mutagenesis and selection for increased formation of epothilone A and/or B over *Sorangium cellulosum Soce90*, which is deposited in the DSM under no. 6773, especially the mutant BCE33/10, which was deposited under the number DSM 11999 on 9th February 1998 at the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany).

Strain culture and morphological description of strain BCE 33/10: The strain can grow on cellulose as the sole source of carbon and energy with potassium nitrate as the sole source of nitrogen, e.g. on filter paper over ST21 mineral salt agar (0.1 % KNO<sub>3</sub>; 0.1 % MgSO<sub>4</sub> x 7

H<sub>2</sub>O; 0.1 % CaCl<sub>2</sub> x 2 H<sub>2</sub>O; 0.1 % K<sub>2</sub>HPO<sub>4</sub>; 0.01 % MnSO<sub>4</sub> x 7 H<sub>2</sub>O; 0.02 % FeCl<sub>3</sub>; 0.002% yeast extract; standard trace element solution; 1% agar). On this medium, dark reddish-brown to blackish-brown fruiting bodies are formed. They consist of small sporangioles (ca. 15 to 30 µm diameter) and exist in dense heaps and packs of varying size.

The vegetative bacilli have the shape typical of *Sorangium* (relatively compact, under the phase contrast microscope dark, cylindrical bacilli with broad rounded ends, on average 3 to 6 µm long and 1 µm thick).

Epothilones are primarily epothilone A and/or ~~B~~, but also other epothilones, for example epothilones C and D named in International Application WO 97/19086 and WO 98/22461, epothilones E and F named in WO 98/22461, and further epothilones obtainable from corresponding microorganisms.

A water-soluble complex-forming component is primarily a water-soluble oligo- or polypeptide derivative or in particular an oligo- or polysaccharide derivative of cyclic or helical structure, which forms an intramolecular cavity, which because of its sufficiently hydrophobic properties is in a position to bind epothilones, especially epothilone A and/or epothilone B. A water-soluble complex-forming component that is especially preferred is one that is selected from cyclodextrins or (in particular) cyclodextrin derivatives, or mixtures thereof.

Cyclodextrins are cyclic (α-1,4)-linked oligosaccharides of α-D-glucopyranose with a relatively hydrophobic central cavity and a hydrophilic external surface area.

The following are distinguished in particular (the figures in parenthesis give the number of glucose units per molecule): α-cyclodextrin (6), β-cyclodextrin (7), γ-cyclodextrin (8), δ-cyclodextrin (9), ε-cyclodextrin (10), ζ-cyclodextrin (11), η-cyclodextrin (12), and θ-cyclodextrin (13). Especially preferred are δ-cyclodextrin and in particular α-cyclodextrin, β-cyclodextrin or γ-cyclodextrin, or mixtures thereof.

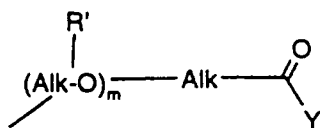
Cyclodextrin derivatives are primarily derivatives of the above-mentioned cyclodextrins, especially of α-cyclodextrin, β-cyclodextrin or γ-cyclodextrin, primarily those in which one or



more up to all of the hydroxy groups (3 per glucose radical) are etherified or esterified. Ethers are primarily alkyl ethers, especially lower alkyl, such as methyl or ethyl ether, also propyl or butyl ether; the aryl-hydroxyalkyl ethers, such as phenyl-hydroxy-lower-alkyl, especially phenyl-hydroxyethyl ether; the hydroxyalkyl ethers, in particular hydroxy-lower-alkyl ethers, especially 2-hydroxyethyl, hydroxypropyl such as 2-hydroxypropyl or hydroxy-butyl such as 2-hydroxybutyl ether; the carboxyalkyl ethers, in particular carboxy-lower-alkyl ethers, especially carboxymethyl or carboxyethyl ether; derivatised carboxyalkyl ethers, in particular derivatised carboxy-lower-alkyl ether in which the derivatised carboxy is etherified or amidated carboxy (primarily aminocarbonyl, mono- or di-lower-alkyl-aminocarbonyl, morpholino-, piperidino-, pyrrolidino- or piperazino-carbonyl, or alkyloxycarbonyl), in particular lower alkyloxycarbonyl-lower-alkyl ether, for example methyloxycarbonylpropyl ether or ethyloxycarbonylpropyl ether; the sulfoalkyl ethers, in particular sulfo-lower-alkyl ethers, especially sulfobutyl ether; cyclodextrins in which one or more OH groups are etherified with a radical of formula



wherein alk is alkyl, especially lower alkyl, and n is a whole number from 2 to 12, especially 2 to 5, in particular 2 or 3; cyclodextrins in which one or more OH groups are etherified with a radical of formula



wherein R' is hydrogen, hydroxy,  $-O-(alk-O)_2-H$ ,  $-O-(alk(-R)-O)_p-H$  or

$-O-(alk(-R)-O)_q-alk-CO-Y$ ; alk in all cases is alkyl, especially lower alkyl; m, n, p, q and z are a whole number from 1 to 12, preferably 1 to 5, in particular 1 to 3; and Y is  $OR_1$  or  $NR_2R_3$ , wherein  $R_1$ ,  $R_2$  and  $R_3$  independently of one another, are hydrogen or lower alkyl, or  $R_2$  and  $R_3$  combined together with the linking nitrogen signify morpholino, piperidino, pyrrolidino or piperazino;

or branched cyclodextrins, in which etherifications or acetals with other sugar molecules are present, especially glucosyl-, diglucosyl- ( $G_2$ - $\beta$ -cyclodextrin), maltosyl- or dimaltosyl-

cyclodextrin, or N-acetylglucosaminyl-, glucosaminyl-, N-acetylgalactosaminyl- or galactosaminyl-cyclodextrin.

Esters are primarily alkanoyl esters, in particular lower alkanoyl esters, such as acetyl esters of cyclodextrins.

It is also possible to have cyclodextrins in which two or more different said ether and ester groups are present at the same time.

Mixtures of two or more of the said cyclodextrins and/or cyclodextrin derivatives may also exist.

Preference is given in particular to  $\alpha$ -,  $\beta$ - or  $\gamma$ -cyclodextrins or the lower alkyl ethers thereof, such as methyl- $\beta$ -cyclodextrin or in particular 2,6-di-O-methyl- $\beta$ -cyclodextrin, or in particular the hydroxy lower alkyl ethers thereof, such as 2-hydroxypropyl- $\alpha$ -, 2-hydroxypropyl- $\beta$ - or 2-hydroxypropyl- $\gamma$ -cyclodextrin.

The cyclodextrins or cyclodextrin derivatives are added to the culture medium preferably in a concentration of 0.02 to 10, preferably 0.05 to 5, especially 0.1 to 4, for example 0.1 to 2 percent by weight (w/v).

Cyclodextrins or cyclodextrin derivatives are known or may be produced by known processes (see for example US 3,459,731; US 4,383,992; US 4,535,152; US 4,659,696; EP 0 094 157; EP 0 149 197; EP 0 197 571; EP 0 300 526; EP 0 320 032; EP 0 499 322; EP 0 503 710; EP 0 818 469; WO 90/12035; WO 91/11200; WO 93/19061; WO 95/08993; WO 96/14090; GB 2,189,245; DE 3,118,218; DE 3,317,064 and the references mentioned therein, which also refer to the synthesis of cyclodextrins or cyclodextrin derivatives, or also: T. Loftsson and M.E. Brewster (1996): Pharmaceutical Applications of Cyclodextrins: Drug Solubilization and Stabilisation: Journal of Pharmaceutical Science 85 (10):1017-1025; R.A. Rajewski and V.J. Stella(1996): Pharmaceutical Applications of Cyclodextrins: In Vivo Drug Delivery: Journal of Pharmaceutical Science 85 (11): 1142-1169).

In the following description of the working up and purification, epothilone is understood to be an epothilone which is obtainable from the corresponding microorganism, preferably epothilone C, D, E, F or especially A or in particular epothilone B. If not otherwise stated, where "epothilones" are mentioned, this is intended to be a general term which includes individual epothilones or mixtures.

Working up of the epothilones is effected by conventional methods; first of all, by separating a culture into the liquid phase (centrifugate or filtrate) and solid phase (cells) by means of filtration or centrifugation (tubular centrifuge or separator). The (main) part of the epothilones found in the centrifugate or in the filtrate is then directly mixed with a synthetic resin, for example a resin based on polystyrene as matrix (hereinafter referred to also simply as polystyrene resin), such as Amberlite XAD-16 [Rohm & Haas Germany GmbH, Frankfurt] or Diaion HP-20 [Resindion S.R.L., Mitsubishi Chemical Co., Milan] (preferably in a ratio of centrifugate: resin volume of ca. 10:1 to 100:1, preferably about 50:1). After a period of contact of preferably 0.25 to 50 hours, especially 0.8 to 22 hours, the resin is separated, for example by filtration or centrifugation. If required, after adsorption, the resin is washed with a strongly polar solvent, preferably with water. Desorption of the epothilones is then effected with an appropriate solvent, especially with an alcohol, in particular isopropanol. The solvent phase, especially isopropanol phase, is then removed from the solvent, preferably by means of prior, simultaneous or subsequent addition of water, in particular in a circulating evaporator, thereby being concentrated if necessary, and the resulting water phase is extracted with a solvent suitable for forming a second phase, such as an ester, for example a lower alkanol lower alkanoate, typically ethyl acetate or isopropyl acetate. The epothilones are thereby transferred into the organic phase. Then the organic phase is concentrated to the extent necessary, preferably to dryness, for example using a rotary evaporator.

Subsequently, further processing takes place using the following steps, whereby the purification step by means of reversed-phase chromatography with elution with a nitrile is an inventive step and is thus compulsory, while the other steps are optional:

-molecular filtration (gel chromatography), e.g. on a column of material such as Sephadex LH-20 (Pharmacia, Uppsala, Sweden) with an alcohol such as methanol as eluant;

- separation of the epothilones by reversed-phase chromatography after being taken up in a suitable solvent, and elution with a mixture of nitrile/water (compulsory), preferably characterised in that the chromatography is carried out on a column of material, especially a RP-18 material, which is charged with hydrocarbon chains, such as hydrocarbon chains containing 18 carbon atoms, and an eluant comprising a nitrile, especially a lower alkyl-nitrile, in particular acetonitrile, is used, in particular a mixture of nitrile/water is used, especially a mixture of acetonitrile/water, preferably in a ratio of nitrile to water of about 1:99 to 99:1, primarily between 1:9 and 9:1, e.g. between 2:8 and 7:3, e.g. 3:7 or 4:6.
- single or multiple extraction of the residue (especially after evaporation) in a two-phase system consisting of water and a solvent immiscible with water, preferably an ester, in particular a lower alkyl lower alkanoate, such as ethyl acetate or isopropyl acetate;
- adsorption chromatography, in particular by adding to a column of silica gel and eluting with an appropriate solvent or solvent mixture, especially a mixture of ester/hydrocarbon, for example lower alkyl alkanoate / C<sub>4</sub>-C<sub>10</sub>-alkane, especially ethyl or isopropyl acetate / n-hexane, in which the ratio between the ester and hydrocarbon is preferably in the range 99:1 to 1:99, preferably 10:1 to 1:10, for example 4:1;
- dissolving the residue, which may be obtained after concentration, in an appropriate solvent such as an alcohol, e.g. methanol;
- mixing with activated carbon and removal thereof;
- recrystallisation, e.g. from appropriate solvents or solvent mixtures, for example consisting of esters, ester/hydrocarbon mixtures or alcohols, especially ethyl or isopropyl acetate : toluene 1:10 to 10:1, preferably 2:3 (epothilone A) or methanol or ethyl acetate (epothilone B);

whereby between each step being employed, the resulting solutions or suspensions are concentrated if necessary, and/or liquid and solid components are separated from one another, in particular by filtering or centrifuging solutions/suspensions. The more precise definitions mentioned below can be preferably used in the above individual steps.

Working up and purification are preferably carried out as follows: After harvest, a culture is separated into the liquid phase (centrifugate) and solid phase (cells) by means of centrifugation (tubular centrifuge or separator). The main part of the epothilones are found in the centrifugate, which is then directly mixed with a polystyrene resin, such as Amberlite XAD-16 [Rohm & Haas Germany GmbH, Frankfurt] or Diaion HP-20 [Resindion S.R.L.,

Mitsubishi Chemical Co., Milan] (preferably in a ratio of centrifugate: resin volume of ca. 10:1 to 100:1, preferably about 50:1) and stirred in an agitator. In this step, the epothilones are transferred from the cyclodextrin to the resin. After a period of contact of ca. 1 hour, the resin is separated by centrifugation or filtration. Adsorption of the epothilones onto the resin may also be effected in a chromatography column, by placing the resin in the column and running the centrifugate over the resin. After adsorption, the resin is washed with water. Desorption of the epothilones from the resin is effected with isopropanol. The isopropanol phase is then freed of isopropanol preferably by the addition of water in particular in a circulating evaporator, and the resulting water phase is extracted with ethyl acetate. The epothilones are transferred from the water phase to the ethyl acetate phase. Then the ethyl acetate extract is concentrated to dryness, using a rotary evaporator. An initial concentration of the epothilones is then achieved by means of molecular filtration (e.g. Sephadex LH-20 [Pharmacia, Uppsala, Sweden] with methanol as eluant). The peak fractions from the molecular filtration contain epothilone A and B and have a total epothilone content of > 10%. Separation of these peak fractions, which contain epothilone A and B in a mixture, into the individual components, then follows by means of chromatography on a "reversed-phase", e.g. RP-18 phase (phase which is modified by alkyl radicals containing 18 carbon atoms per chain), with an appropriate eluant, preferably one containing a nitrile such as acetonitrile (this gives better separation than for example alcohols such as methanol). Epothilone A elutes before epothilone B. The peak fractions with epothilone B may still contain small portions of epothilone A, which can be removed by further separation on RP-18. Finally, the epothilone A fraction is crystallised directly from ethyl acetate:toluene = 2:3, and the epothilone B fraction from methanol or ethyl acetate.

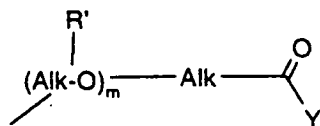
#### Preferred embodiment of the invention

The invention preferably relates to a process for the concentration of epothilones, especially epothilone A and/or B, in particular epothilone B, in a culture medium for the biotechnological preparation of this (these) compound(s), which contains a microorganism which is suitable for this preparation, especially a *Sorangium* strain, especially of the type *Sorangium Cellulosum Soce90*, or a mutant arising therefrom, in particular the strain having reference BCE 33/10, water and other usual appropriate constituents of culture media, whereby a cyclodextrin or a cyclodextrin derivative, or a mixture of two or more of these compounds is added to the medium, especially one or more, preferably one or two or more

cyclodextrins selected from  $\alpha$ -cyclodextrin (6),  $\beta$ -cyclodextrin (7),  $\gamma$ -cyclodextrin (8),  $\delta$ -cyclodextrin (9),  $\epsilon$ -cyclodextrin (10),  $\zeta$ -cyclodextrin (11),  $\eta$ -cyclodextrin (12), and  $\theta$ -cyclodextrin (13), especially  $\alpha$ -cyclodextrin,  $\beta$ -cyclodextrin or  $\gamma$ -cyclodextrin; or primarily a cyclodextrin derivative or mixture of cyclodextrin derivatives selected from derivatives of a cyclodextrin, in which one or more up to all of the hydroxy groups are etherified to an alkyl ether, especially lower alkyl, such as methyl or ethyl ether, also propyl or butyl ether; an aryl-hydroxyalkyl ether, such as phenyl-hydroxy-lower-alkyl, especially phenyl-hydroxyethyl ether; a hydroxyalkyl ether, in particular hydroxy-lower-alkyl ether, especially 2-hydroxyethyl, hydroxypropyl such as 2-hydroxypropyl or hydroxybutyl such as 2-hydroxybutyl ether; a carboxyalkyl ether, in particular carboxy-lower-alkyl ether, especially carboxymethyl or carboxyethyl ether; a derivatised carboxyalkyl ether, in particular a derivatised carboxy-lower-alkyl ether in which the derivatised carboxy is aminocarbonyl, mono- or di-lower-alkyl-aminocarbonyl, morpholino-, piperidino-, pyrrolidino- or piperazino-carbonyl, or alkyloxycarbonyl, in particular lower alkyloxycarbonyl, such as preferably a lower alkoxy-carbonyl-lower-alkyl ether, for example methyloxycarbonylpropyl ether or ethyloxycarbonylpropyl ether; a sulfoalkyl ether, in particular sulfo-lower-alkyl ether, especially sulfobutyl ether; a cyclodextrin in which one or more OH groups are etherified with a radical of formula



wherein alk is alkyl, especially lower alkyl, and n is a whole number from 2 to 12, especially 2 to 5, in particular 2 or 3; a cyclodextrin in which one or more OH groups are etherified with a radical of formula



wherein R' is hydrogen, hydroxy,  $-O-(alk-O)_2-H$ ,  $-O-(alk(-R)-O)_p-H$  or  $-O-(alk(-R)-O)_q-alk-CO-Y$ ; alk in all cases is alkyl, especially lower alkyl; m, n, p, q and z are a whole number from 1 to 12, preferably 1 to 5, in particular 1 to 3; and Y is  $OR_1$  or  $NR_2R_3$ , wherein  $R_1$ ,  $R_2$  and  $R_3$  independently of one another, are hydrogen or lower alkyl, or  $R_2$  and

R<sub>3</sub> combined together with the linking nitrogen signify morpholino, piperidino, pyrrolidino or piperazino;  
or a branched cyclodextrin, in which etherifications or acetals with other sugar molecules are present, and which are selected from glucosyl-, diglucosyl- (G<sub>2</sub>-β-cyclodextrin), maltosyl- or dimaltosyl-cyclodextrin, or N-acetylglucosaminy-, glucosaminy-, N-acetylgalactosaminy- and galactosaminy-cyclodextrin; or a lower alkanoyl, such as acetyl ester of a cyclodextrin.

Particular preference is given to a process in which the cyclodextrin and/or the cyclodextrin derivative is added to the culture medium in a concentration of 0.02 to 10, preferably 0.005 to 10, more preferably 0.05 to 5, most preferably 0.1 to 4, for example 0.1 to 2, percent by weight (w/v).

Especially preferred is a process according to one of the two previous paragraphs, in which the cyclodextrin derivative is selected from a cyclodextrin, especially β-cyclodextrin, and a hydroxy lower alkyl-cyclodextrin, especially 2-hydroxypropyl- α -, -β- or -γ-cyclodextrin; or mixtures of one or more thereof; whereby 2-hydroxypropyl-β-cyclodextrin on its own is preferred in particular.

The invention also relates in particular to a culture medium, which comprises a cyclodextrin, a cyclodextrin derivative or a mixture of two or more complex-forming components selected from cyclodextrins and cyclodextrin derivatives, especially a cyclodextrin or cyclodextrin derivative as defined in the third-last paragraph, in particular as in the second-last paragraph, or a mixture of one or more of these compounds, and a microorganism which is suitable for producing epothilones, especially epothilone A and/or B, preferably a strain from the genus *Sorangium*, especially a strain of *Sorangium Cellulosum*, e.g. the strain *Soce90* or a mutant arising therefrom, in particular the strain BCE 33/10.

A further aspect of the invention relates to a process for the production of epothilone A and/or B, especially the two pure compounds, in particular epothilone B, which is characterised in that the epothilones are separated for example by centrifugation into the solid and the liquid phase (centrifugate) by working up a culture medium for the biotechnological preparation of these compounds, as described above, to which has been added a complex-forming component which is soluble in the culture medium, in particular a

cyclodextrin, a cyclodextrin derivative or a mixture of two or more cyclodextrins and/or cyclodextrin derivatives; the centrifugate is mixed with a resin, especially a polystyrene resin, or is run through a column filled with such a resin; if necessary, the resin is washed with water; the epothilone(s) is or are desorbed from the resin using a polar solvent, especially an alcohol, primarily a lower alkanol such as isopropanol; if necessary, concentrated by means of prior, simultaneous or subsequent addition of water; an organic solvent which is immiscible with water, for example an ester, such as ethyl acetate, is added, and the epothilone(s) is or are transferred to the organic phase, for example by agitating or stirring; where necessary, the organic phase is concentrated; the epothilones from the organic solution obtained are concentrated through a molecular sieve for compounds of low molecular weight; and then the fractions containing the epothilones, especially epothilone A and/or B undergo separation by a reversed-phase column, preferably eluting with an eluant containing a nitrile, such as acetonitrile (or instead, an eluant containing an alcohol, such as methanol); whereby epothilones A and B are extracted separately, and if desired, can be further concentrated by recrystallisation.

A further preferred aspect of the invention relates to a method of separating epothilones, especially epothilones A and B from one another, which is characterised by chromatography on a reversed-phase column with an eluant containing a lower alkyl cyanide, chromatography being carried out on a column material, especially an RP-18 material, which is charged with hydrocarbon chains, such as those containing 18 carbon atoms, and employing an eluant containing a nitrile, especially a lower alkyl nitrile, in particular acetonitrile, especially a mixture of nitrile/water, in particular a mixture of acetonitrile/water, preferably in a ratio of nitrile to water of ca. 1:99 to 99:1, primarily 1:9 to 9:1, e.g. between 2:8 and 7:3, e.g. 3:7 or 4:6. This separation may follow on to a filtration with a molecular sieve, or is preferably effected directly using the residue after adsorption of the epothilones from the culture medium containing a complex-forming component onto a resin. One advantage of separation with an eluant containing a lower alkyl cyanide over that using alcohols, such as methanol, is the better separation of epothilones A and B.

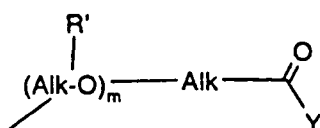
The invention relates preferably to a process for the preparation of epothilones, which a) comprises a process for the concentration of epothilones, especially epothilone A and/or B, in particular epothilone B, in a culture medium for the biotechnological preparation of this (these) compound(s), which contains a microorganism which is suitable for this preparation,



especially a *Sorangium* strain, especially of the type *Sorangium Cellulosum* Soce90, or a mutant arising therefrom, in particular the strain having reference BCE 33/10, water and other usual appropriate constituents of culture media, whereby a cyclodextrin or a cyclodextrin derivative, or a mixture of two or more of these compounds is added to the medium, especially one or more, preferably one or two or more cyclodextrins selected from  $\alpha$ -cyclodextrin (6),  $\beta$ -cyclodextrin (7),  $\gamma$ -cyclodextrin (8),  $\delta$ -cyclodextrin (9),  $\epsilon$ -cyclodextrin (10),  $\zeta$ -cyclodextrin (11),  $\eta$ -cyclodextrin (12), and  $\theta$ -cyclodextrin (13), especially  $\alpha$ -cyclodextrin,  $\beta$ -cyclodextrin or  $\gamma$ -cyclodextrin; or primarily a cyclodextrin derivative or mixture of cyclodextrin derivatives selected from derivatives of a cyclodextrin, in which one or more up to all of the hydroxy groups are etherified to an alkyl ether, especially lower alkyl, such as methyl or ethyl ether, also propyl or butyl ether; an aryl-hydroxyalkyl ether, such as phenyl-hydroxy-lower-alkyl, especially phenyl-hydroxyethyl ether; a hydroxyalkyl ether, in particular hydroxy-lower-alkyl ether, especially 2-hydroxyethyl, hydroxypropyl such as 2-hydroxypropyl or hydroxybutyl such as 2-hydroxybutyl ether; a carboxyalkyl ether, in particular carboxy-lower-alkyl ether, especially carboxymethyl or carboxyethyl ether; a derivatised carboxyalkyl ether, in particular a derivatised carboxy-lower-alkyl ether in which the derivatised carboxy is aminocarbonyl, mono- or di-lower-alkyl-aminocarbonyl, morpholino-, piperidino-, pyrrolidino- or piperazino-carbonyl, or alkyloxycarbonyl, in particular lower alkyloxycarbonyl, such as preferably a lower alkyloxycarbonyl-lower-alkyl ether, for example methyloxycarbonylpropyl ether or ethyloxycarbonylpropyl ether; a sulfoalkyl ether, in particular sulfo-lower-alkyl ether, especially sulfobutyl ether; a cyclodextrin in which one or more OH groups are etherified with a radical of formula



wherein alk is alkyl, especially lower alkyl, and n is a whole number from 2 to 12, especially 2 to 5, in particular 2 or 3; a cyclodextrin in which one or more OH groups are etherified with a radical of formula



wherein R' is hydrogen, hydroxy, -O-(alk-O)<sub>z</sub>-H, -O-(alk(-R)-O)<sub>p</sub>-H or -O-(alk(-R)-O)<sub>q</sub>-alk-CO-Y; alk in all cases is alkyl, especially lower alkyl; m, n, p, q and z are a whole number from 1 to 12, preferably 1 to 5, in particular 1 to 3; and Y is OR<sub>1</sub> or NR<sub>2</sub>R<sub>3</sub>, wherein R<sub>1</sub>, R<sub>2</sub> and R<sub>3</sub> independently of one another, are hydrogen or lower alkyl, or R<sub>2</sub> and R<sub>3</sub> combined together with the linking nitrogen signify morpholino, piperidino, pyrrolidino or piperazino;

or a branched cyclodextrin, in which etherifications or acetals with other sugar molecules are present, and which are selected from glucosyl-, diglucosyl- (G<sub>2</sub>-β-cyclodextrin), maltosyl- or dimaltosyl-cyclodextrin, or N-acetylglucosaminy-, glucosaminy-, N-acetylgalactosaminy- and galactosaminy-cyclodextrin; or a lower alkanoyl, such as acetyl ester of a cyclodextrin; and

b) comprises a step for separating the epothilones, especially epothilones A and B, from one another, which is characterised by chromatography on a reversed-phase column with an eluant containing a lower alkylcyanide, the chromatography being carried out on a column material, especially an RP-18 material, which is charged with hydrocarbon chains, such as those containing 18 carbon atoms, and employing an eluant containing a lower alkyl nitrile, especially acetonitrile, in particular a mixture of lower alkyl nitrile/water, preferably a mixture of acetonitrile/water, preferably in a ratio of lower alkyl nitrile to water of ca. 1:99 to 99:1, primarily 1:9 to 9:1, e.g. between 2:8 and 7:3, e.g. 3:7 or 4:6, whereby if desired, it is possible to use further steps for working up and purification.

The invention also relates in particular to a mutant derived from the strain *Sorangium cellulosum* Soce90, especially a strain of *Sorangium cellulosum* which is obtainable by mutagenesis, preferably by one or more UV-induced mutagenesis steps (in particular by UV radiation in the range 200 to 400, especially 250 to 300 nm) with subsequent searching in each step for mutants having increased epothilone production (in particular increased epothilone concentration in the culture medium), this strain under otherwise identical conditions producing more epothilones, in particular more epothilone A and/or B, especially epothilone B, than *Sorangium cellulosum* Soce90, especially the *Sorangium cellulosum* strain BCE 33/10.

The invention relates in particular to the individual process steps named in the examples or any combination thereof, the culture media named therein, crystal forms and the strain described therein.

The invention also relates to new crystal forms of epothilone B, especially a crystal form of epothilone B described as modification B and in particular described as modification A.

The crystal forms can be distinguished in particular by their X-ray diagrams. X-ray diagrams taken with a diffractometer and using Cu-K $\alpha_1$ -radiation are preferably used to characterise solids of organic compounds. X-ray diffraction diagrams are used particularly successfully to determine the crystal modification of a substance. To characterise the existing crystal modification A and crystal modification B of epothilone B, the measurements are made at an angle range ( $2\theta$ ) of  $2^\circ$  and  $35^\circ$  with samples of substance that are kept at room temperature.

The X-ray diffraction diagram thus determined (reflection lines and intensities of the most important lines) from crystal modification A (modification A) of epothilone B is characterised by the following table.

| $2\theta$ | Intensity   |
|-----------|-------------|
| 7.7       | very strong |
| 10.6      | weak        |
| 13.6      | average     |
| 14.4      | average     |
| 15.5      | average     |
| 16.4      | weak        |
| 16.8      | weak        |
| 17.1      | weak        |
| 17.3      | weak        |
| 17.7      | weak        |
| 18.5      | weak        |
| 20.7      | strong      |
| 21.2      | strong      |

|      |         |
|------|---------|
| 21.9 | weak    |
| 22.4 | weak    |
| 23.3 | strong  |
| 25.9 | average |
| 31.2 | weak    |
| 32.0 | average |

The invention also relates in particular to a new crystal form of epothilones B, which is characterised by a melting point of more than 120 °C, especially between 120 °C and 128°C, in particular 124-125 °C. Surprisingly, this value is considerably higher than the values previously described in literature. The invention relates especially to a crystal form of epothilone B, which is characterised by the X-ray diffraction diagram of the crystal form A and a melting point of above 120°C, especially between 120°C and 128 °C, for example between 124 °C and 125 °C.

The X-ray diffraction diagram thus determined (reflection lines and intensities of the most important lines) from crystal modification B (modification B) of epothilone B is characterised by the following table.

| 2θ   | Intensity   |
|------|-------------|
| 6.9  | very strong |
| 8.0  | weak        |
| 8.3  | average     |
| 10.8 | strong      |
| 11.5 | average     |
| 12.4 | weak        |
| 13.1 | strong      |
| 15.5 | weak        |
| 16.2 | weak        |
| 16.7 | average     |
| 18.1 | average     |
| 18.6 | average     |
| 20.4 | weak        |

|      |           |
|------|-----------|
| 20.9 | strong    |
| 21.3 | weak      |
| 21.5 | very weak |
| 22.5 | average   |
| 24.2 | weak      |
| 25.1 | average   |

The new crystal forms are especially stable, in particular crystal form A is to be regarded as the one which is more thermodynamically stable, and they are therefore suitable as active ingredients for solid forms of administration, for storing in solid form or as intermediates (with particularly good storability) in the preparation of solid or liquid forms of administration.

The invention also relates to the use of the new crystal forms, especially crystal form B, but primarily crystal form A (all referred to hereinafter as active ingredient) in the production of pharmaceutical preparations, new pharmaceutical preparations which contain these new crystal forms, and/or their use in the treatment of proliferative diseases, such as tumours. In the following, where pharmaceutical preparations or compositions which comprise or contain the active ingredient are mentioned, in the case of liquid compositions or compositions which no longer contain the crystal form as such, this is always understood to mean also the pharmaceutical preparations obtainable using the crystal forms (for example infusion solutions obtained using crystal forms A or B of epothilone B), even if they no longer contain the respective crystal form (for example because they exist in solution).

The invention also relates especially to the use of a new crystal form of epothilone B, especially the crystal form B or in particular crystal form A, in the production of pharmaceutical preparations, characterised by mixing a new crystal form of epothilone B with one or more carriers.

The invention also relates to a method of treating warm-blooded animals suffering from a proliferative disease, characterised by administering a dose of epothilone B which is effective for treating said disease in one or the new crystal forms to a warm-blooded animal requiring such treatment, also including in particular the treatment with those preparations that are produced using one of the new crystal forms; and/or the use of a new crystal form of epothilone B in such a treatment.

To produce the pharmaceutical preparations, the active ingredient may be used for example in such a way that the pharmaceutical preparations contain an effective amount of the active ingredient together or in a mixture with a significant amount of one or more organic or inorganic, liquid or solid, pharmaceutically acceptable carriers.

The invention also relates to a pharmaceutical composition which is suitable for administration to a warm-blooded animal, especially humans, in the treatment of a proliferative disease, such as a tumour, the composition containing an amount of active ingredient that is suitable for treating said disease, together with a pharmaceutically acceptable carrier.

The pharmaceutical compositions according to the invention are those intended for enteral, especially nasal, rectal or oral, or preferably parenteral, especially intramuscular or intravenous administration to warm-blooded animals, especially humans, and they contain an effective dose of the active ingredient on its own or together with a significant amount of a pharmaceutically acceptable carrier. The dose of the active ingredient is dependent on the type of warm-blooded animal, the body weight, the age and the individual condition, individual pharmacokinetic situations, the disease to be treated and the type of administration.

The pharmaceutical compositions contain ca. 0.0001 % to ca. 95 %, preferably 0.001 % to 10 % or 20 % to ca. 90 % of active ingredient. Pharmaceutical compositions according to the invention may be present for example in unit dose forms, such as in the form of ampoules, ~~viats~~, suppositories, dragées, tablets or capsules.

The pharmaceutical compositions according to the present invention are produced by known processes, for example by conventional dissolving, lyophilising, mixing, granulating or manufacturing processes.

Solutions of the active ingredient, also suspensions, and in particular aqueous solutions or suspensions, are preferably employed, whereby it is also possible, for example in the case of lyophilised compositions which contain the active ingredient on its own or together with a pharmaceutically acceptable carrier, for example mannitol, for the solutions or suspensions

to be prepared prior to administration. The pharmaceutical compositions may be sterilised and/or may contain excipients, for example preservatives, stabilisers, moisture-retaining agents and/or emulsion-forming agents, dissolving aids, salts for regulating osmotic pressure and/or buffers, and they are produced by known processes, for example by conventional dissolving or lyophilising processes. The solutions or suspensions mentioned may comprise viscosity-increasing substances, such as sodium carboxymethylcellulose, carboxymethylcellulose, dextran, polyvinylpyrrolidone or gelatin.

Suspensions in oil contain as the oil component vegetable oils, synthetic oils or semi-synthetic oils, which are customary for injection purposes. Notable examples are in particular liquid fatty acid esters, which contain as the acid component a long-chained fatty acid with 8 to 22, especially 12 to 22, carbon atoms, for example lauric acid, tridecylic acid, myristic acid, pentadecylic acid, palmitic acid, margaric acid, stearic acid, arachidic acid, behenic acid or corresponding unsaturated acids, for example oleic acid, elaidic acid, erucic acid, brassidic acid or linoleic acid, if desired with the addition of antioxidants, for example vitamin E,  $\beta$ -carotene or 3,5-di-tert-butyl-4-hydroxytoluene. The alcoholic component of these fatty acid esters preferably has a maximum of 6 carbon atoms and is a mono- or polyhydroxy alcohol, for example a mono-, di- or tri-hydroxy alcohol, for example methanol, ethanol, propanol, butanol or pentanol, or an isomer thereof, but especially glycol and glycerol. The following examples of fatty acid esters may be mentioned in particular: propyl myristate, isopropyl palmitate, "Labrafil M 2375" (polyoxyethylene glycerol trioleate, Gattefossé, Paris), "Miglyol 812" (triglyceride of saturated fatty acids having a chain length of 8 to 12 carbon atoms, Hüls AG, Germany), but in particular vegetable oils such as cottonseed oil, almond oil, olive oil, castor oil, sesame oil, soybean oil and in particular peanut oil.

The injection or infusion preparations are produced according to customary methods under sterile conditions; the same applies also to the filling of the compositions into ampoules or vials and sealed containers.

Preference is given to an infusion solution which contains the active ingredient and a pharmaceutically acceptable organic solvent.

The pharmaceutically acceptable organic solvents which may be used in a formulation according to the invention can be selected from all such solvents which are familiar to a person skilled in the art. The solvent is preferably selected from an alcohol, e.g. absolute ethanol, ethanol/water mixtures, preferably 70 % ethanol, polyethylene glycol 300, polyethylene glycol 400, polypropylene glycol and N-methylpyrrolidone, especially polypropylene glycol or 70 % ethanol.

The active ingredient is present in the formulation in a concentration of 0.001 to 100 mg/ml, preferably from ca. 0.05 to 5 mg/ml, or from 5 to 50 mg/ml.

Formulations of this type are easily stored as vials or ampoules. The vials or ampoules are typically made of glass, e.g. boron silicate. The vials or ampoules may be appropriate for any volume which is known from the prior art. They are preferably of sufficient size to be able to accept 0.5 to 5 ml of the formulation.

Prior to administration, the formulations have to be diluted in an aqueous medium suitable for intravenous administration before the active ingredient can be administered to patients.

It is preferable for the infusion solution to have the same or basically the same osmotic pressure as body fluids. Consequently, the aqueous medium contains an isotonic agent which has the effect of rendering the osmotic pressure of the infusion solution the same or basically the same as the osmotic pressure of body fluids.

The isotonic agent can be selected from all agents that are familiar to a person skilled in the art, for example mannitol, dextrose, glucose and sodium chloride. The isotonic agent is preferably glucose or sodium chloride. The isotonic agents may be used in quantities which impart the same or basically the same osmotic pressure to the infusion solution as body fluids. The exact quantities required can be determined by routine experiments and depend on the composition of the infusion solution and the type of isotonic agent.

The concentration of isotonizing agent in the aqueous medium depends on the type of each agent used. If glucose is used, it is preferably used in a concentration of 1 to 5% w/v, preferably 5% w/v. If the isotonizing agent is sodium chloride, it is preferably used in quantities of up to 1%, preferably ca. 0.9% w/v.



The infusion solution can be diluted with the aqueous medium. The amount of aqueous medium used is chosen according to the desired concentration of active ingredient in the infusion solution. The infusion solution is preferably produced by mixing a vial or an ampoule containing the infusion concentrate (see above) with an aqueous medium, so that a volume of between 200 ml and 1000 ml is attained with the aqueous medium. Infusion solutions may contain other additives that are normally used in formulations for intravenous administration. These additives also include antioxidants.

Antioxidants may be used to protect the active ingredient from degradation by oxidation. Antioxidants may be selected from those which are familiar to the person skilled in the art and which are suitable for intravenous formulations. The amount of antioxidant can be determined by routine experiments. As an alternative to adding an antioxidant, or additionally thereto, the antioxidant effect can be achieved by restricting the oxygen (air) contact with the infusion solution. This can be achieved in a simple way, by treating the vessel containing the infusion solution with an inert gas, e.g. nitrogen or argon.

Infusion solutions can be produced by mixing an ampoule or a vial with the aqueous medium, e.g. a 5% glucose solution in WFI in an appropriate container, e.g. an infusion bag or an infusion bottle.

Containers for the infusion solutions may be selected from conventional containers that are non-reactive with the infusion solution. Among those suitable are glass containers, especially of boron silicate, but plastic containers such as plastic infusion bags, are preferred.

Plastic containers may also be made of thermoplastic polymers. The plastic materials may also contain additives, e.g. softeners, fillers, antioxidants, antistatic agents or other customary additives.

Suitable plastics for the present invention should be resistant to elevated temperatures used for sterilisation. Preferred plastic infusion bags are the PVC materials which are known to the person skilled in the art.

A large range of container sizes may be considered. When selecting the size of the container, the factors to be taken into consideration are especially the solubility of epothilones in an aqueous medium, easy handling, and if appropriate, storage of the container. It is preferable to use containers which hold between ca. 200 and 1000 ml of infusion solution.

Owing to their good formulating properties, the new crystal forms of epothilone B according to the invention are especially suitable for the simple and reproducible production of the said infusion solutions. However, the new crystal forms are especially suitable for the production of pharmaceutical formulations which contain the active ingredient in solid form, for example oral formulations.

Pharmaceutical formulations for oral application may be obtained by combining the active ingredient with solid carriers, if desired by granulating the resultant mixture, and further processing the mixture, if desired or if necessary, after adding suitable adjuvants, into tablets, dragée cores or capsules. It is also possible to embed them in plastic substrates which enable the active ingredient to be diffused or released in measured quantities.

Suitable pharmaceutically employable carriers are especially fillers, such as lactose, saccharose, mannitol or sorbitol, cellulose preparations, and/or calcium phosphates, for example tricalcium phosphate or calcium hydrogen phosphate, and binders, such as starches, for example maize, wheat, rice or potato starch, gelatin, tragacanth, methyl cellulose, hydroxypropyl methyl cellulose, sodium carboxymethylcellulose, and/or polyvinyl pyrrolidone, and/or, if desired, disintegrators, such as the above-mentioned starches, crosslinked vinylpyrrolidones, agar, alginic acid or a salt thereof, such as sodium alginate. Adjuvants are in particular flow-improving agents and lubricants, e.g. silicates, talcum, stearic acid or salts thereof, such as magnesium or calcium stearate and/or polyethylene glycol. Dragée cores are provided, if desired, with appropriate gastric-juice-resistant coatings, using *inter alia* concentrated sugar solutions, gum arabic, talcum, polyvinyl pyrrolidone, polyethylene glycol and/or titanium dioxide, or coating solutions in suitable organic solvents, or in order to produce gastric-juice-resistant coatings, solutions of appropriate cellulose preparations, such as ethyl cellulose phthalate or hydroxypropyl methyl cellulose phthalate. Capsules are dry capsules consisting of gelatin or pectin, and if

required, a softener such as glycerol or sorbitol. The dry capsules may contain the active ingredient in the form of granules, for example with fillers, such as lactose, binders, such as starches, and/or lubricants, such as talc or magnesium stearate, and where appropriate stabilisers. In soft capsules, the active ingredient may be present in dissolved or preferably suspended form, whereby oily adjuvants such as fat oils, paraffin oil or liquid propylene glycols are added; stabilisers and/or antibacterial additives may also be added. Dyes or pigments can be added to the tablets or dragée coatings, for example to improve identification or to distinguish different dosages of active ingredient.

The usage in the treatment of a proliferative disease with one of the crystal forms B and in particular A preferably takes place whereby the crystal form (preferably as for the usage in the preparation of an infusion solution, as described above) is administered to a warm-blooded animal, especially a human, in a dose which can be determined at between 20 and 133%, preferably between 25 and 100%, of the Maximum Tolerated Dose (MTD) by standard methods, for example using a modified Fibonacci series, in which the increases in dosages for successive amounts are 100%, 67%, 50% and 40% followed by 33% for all subsequent doses; and, if necessary, one or more further doses administered in the dosage range given above for the first dose, each dose after a period of time which allows sufficient recovery of the individual being treated after the preceding administration, in particular one week or more after the first administration, preferably 2 to 10 weeks, especially 3 to 6 weeks after each preceding administration. In general, this treatment scheme, in which a high dosage is administered once, twice or several times with sufficiently long intervals between the individual administrations for recovery to take place, is preferred over a more frequent treatment with lower doses, since hospitalisation is less frequent and for a shorter period and an improved anti-tumour effect can be expected. The dosage of epothilone B for humans is preferably between 0.1 and 50 mg/m<sup>2</sup>, preferably between 0.2 and 10 mg/m<sup>2</sup>.

The following Examples serve to illustrate the invention without limiting its scope.

Caution: When handling epothilones, appropriate protective measures must be taken, where necessary, in view of their high toxicity.

The 750 litre fermenter used in the following is a refined steel fermenter from the company Alpha AG, Nidau, Switzerland.

Example 1: Preparation of the strain BCE33/10 by means of mutation and selection

The strain employed is the mutant BCE33/10 (deposited at the German Collection of Microorganisms and Cell Cultures under number DSM 11999 on 9th February 1998), which is derived from the strain *Sorangium cellulosum* Soce90 by mutation and selection as described below. In liquid media, the mutant BCE33/10 forms bacilli typical of *Sorangia*, with rounded ends and a length of 3-6  $\mu\text{m}$ , as well as a width of ca. 1  $\mu\text{m}$ . *Sorangium cellulosum* Soce90 was obtained from the German Collection of Microorganisms under number DSM 6773.

Preparation of the mutant BCE33/10 comprises three mutation steps with UV light and selections of individual colonies. The procedure in detail is carried out in accordance with the following operating steps

Cultivation from the ampoule: The cells of the DSM6773 ampoule are transferred to 10 ml of G52 medium in a 50 ml Erlenmeyer flask and incubated for 6 days in an agitator at 30°C and at 180 rpm. 5 ml of this culture are transferred to 50 ml of G52 medium (in a 200 ml Erlenmeyer flask) and incubated at 180 rpm for 3 days in an agitator at 30°C.

First UV mutation step and selection: Portions of 0.1 ml of the above culture are plated out onto several Petri dishes containing agar medium S42. The plates are then each exposed to UV light (maximum radiation range of 250 - 300 nm) for 90 or 120 seconds at 500  $\mu\text{watt}$  per  $\text{cm}^2$ . The plates are then incubated for 7-9 days at 30°C, until individual colonies of 1-2 mm are obtained. The cells of 100-150 colonies are then each plated out from an individual colony by means of plastic loop in sectors onto Petri dishes containing S42 agar (4 sectors per plate) and incubated for 7 days at 30°C. The cells that have grown on an area of ca. 1  $\text{cm}^2$  agar are transferred by a plastic loop to 10 ml of G52 medium in a 50 ml Erlenmeyer flask and incubated for 7 days at 180 rpm in an agitator at 30°C. 5 ml of this culture are transferred to 50 ml of G52 medium (in a 200 ml Erlenmeyer flask) and incubated at 180 rpm for 3 days in an agitator at 30°C. 10 ml of this culture are transferred to 50 ml of 23B3 medium and incubated for 7 days at 180 rpm in an agitator at 30°C.

To determine the amounts of epothilone A and epothilone B formed in this culture, the following procedure is followed. The 50 ml culture solution is filtered through a nylon sieve (150  $\mu\text{m}$  pore size), and the polystyrene resin Amberlite XAD16 retained on the sieve is rinsed with a little water and subsequently added together with the filter to a 50 ml centrifuge tube (Falcon Labware, Becton Dickinson AG Immengasse 7, 4056 Basle). 10 ml of isopropanol (>99%) are added to the tube with the filter. Afterwards, the well-sealed tube is shaken for 1 hour at 180 rpm in order to dissolve the epothilone A and B, which is bonded to the resin, in the isopropanol. Subsequently, 1.5 ml of the liquid is centrifuged, and ca. 0.8 ml of the supernatant is added using a pipette to a HPLC tube. The HPLC analysis of these samples is effected as described below under HPLC analysis in the product analysis section. The HPLC analysis determines which culture contains the highest content of epothilone B. From the above-described sector plate of the corresponding colony (the plates have been stored at 4°C in the meantime), cells from ca. 1 cm<sup>2</sup> of agar area are transferred by a plastic loop to 10 ml of G52 medium in a 50 ml Erlenmeyer flask and are incubated for 7 days at 180 rpm in an agitator at 30°C. 5 ml of this culture are transferred to 50 ml of G52 medium (in a 200 ml Erlenmeyer flask) and incubated at 180 rpm for 3 days in an agitator at 30°C.

Second and third UV mutation step and selection: The procedure is exactly the same as described above for the first UV mutation step, whereby the selected culture of the best colony from the first UV mutation is used for the second mutagenesis. For the third mutagenesis, the culture of the best colony from the second mutagenesis is used accordingly. The best colony after this third cycle of UV mutation steps, followed by selection of the resulting strains for improved epothilone B production, corresponds to mutant BCE33/f0.

#### Preservation of the strain

10 ml of a 3 day old culture in G52 medium (50 ml medium in a 200 ml Erlenmeyer flask, 30°C and 180 rpm) are transferred to 50 ml of 23B3 medium (in a 200 ml Erlenmeyer flask) and incubated for 3 days at 180 rpm in an agitator at 30°C. 1 ml portions of this culture are removed in a form which is as homogeneous as possible (prior to each removal the culture is shaken by hand in the Erlenmeyer flask) together with the polystyrene resin Amberlite XAD16 (polystyrene adsorption resin, Rohm & Haas, Frankfurt, Germany), then filled into

1.8 ml Nunc cryotubes (A/S Nunc, DK 4000 Roskilde, Denmark) and stored either at -70°C or in liquid nitrogen.

Cultivation of the strains from these ampoules is effected by heating them in the air to room temperature, and subsequently transferring the entire content of the cryo tube to 10 ml G52 medium in an 50 ml Erlenmeyer flask and incubating for 5-7 days at 180 rpm in an agitator at 30°C.

### Media

#### G52 Medium:

|   |        |
|---|--------|
| yeast extract, low in salt (Springer, Maisons-Alfort, France) | 2 g/l  |
| MgSO <sub>4</sub> (7 H <sub>2</sub> O)                        | 1 g/l  |
| CaCl <sub>2</sub> (2 H <sub>2</sub> O)                        | 1 g/l  |
| soya meal defatted (Mucedola S.r.l., Settimo Milan, Italy)    | 2 g/l  |
| potato starch Noredux (Blattmann, Wädenswil, Switzerland)     | 8 g/l  |
| glucose anhydrous   | 2 g/l  |
| Fe-EDTA 8g/l (Product No. 03625, Fluka Chemie AG, CH)         | 1 ml/l |
| pH 7,4, corrected with KOH                                    |        |
| Sterilisation: 20 mins. 120 °C                                |        |

S42 Agar-Medium: as described S. Jaoua et al. Plasmid 28, 157-165 (1992)

|                     |  |    |     |
|---------------------|--|----|-----|
| <u>23B3 Medium:</u> | glucose  | 2  | g/l |
|                     | potato starch Noredux (Blattmann, Wädenswil, Switzerland)  | 20 | g/l |
|                     | soya meal defatted (Mucedola S.r.l., Settimo Milan, Italy) | 16 | g/l |
|                     | Fe-EDTA (Product No. 03625, Fluka, Buchs, Switzerland)     | 8  | g/l |
|                     | HEPES Fluka, Buchs, Switzerland                            | 5  | g/l |
|                     | polystyrene resin XAD16 (Rohm and Haas)                    | 2% | v/v |
|                     | H <sub>2</sub> O deionised                                 |    |     |
|                     | correction of pH to 7.8 with NaOH                          |    |     |
|                     | sterilisation for 20 mins. at 120°C                        |    |     |

(HEPES = 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid)

Example 2: Cultivation in order to produce the epothilones

Strain: *Sorangium cellulosum* Soce-90 BCE 33/10

(Example 1)

Preservation of the strain: In liquid N<sub>2</sub>, as in Example 1.

Media: Precultures and intermediate cultures: **G52**

Main culture: **1B12**

G52 Medium:

yeast extract, low in salt (BioSpringer, Maison Alfort, France) 2 g/l  
MgSO<sub>4</sub> (7 H<sub>2</sub>O) 1 g/l  
CaCl<sub>2</sub> (2 H<sub>2</sub>O) 1 g/l  
soya meal defatted Soyamine 50T (Lucas Meyer, Hamburg,  
Germany) 2 g/l  
potato starch Noredux A-150 (Blattmann, Waedenswil,  
Switzerland) 8 g/l  
glucose anhydrous 2 g/l  
EDTA-Fe(III)-Na salt (8 g/l) 1 ml/l  
pH 7,4, corrected with KOH  
Sterilisation: 20 mins. 120 °C

1B12 Medium:

potato starch Noredux A-150 (Blattmann, Waedenswil,  
Switzerland) 20 g/l  
soya meal defatted Soyamine 50T (Lucas Meyer, Hamburg,  
Germany) 11 g/l  
EDTA-Fe(III)-Na salt 8 mg/l  
pH 7.8, corrected with KOH  
Sterilisation: 20 mins. 120 °C

Addition of cyclodextrins and cyclodextrin derivatives:

Cyclodextrins (Fluka, Buchs, Switzerland, or Wacker Chemie, Munich, Germany) in different concentrations are sterilised separately and added to the 1B12 medium prior to seeding.

Cultivation: 1 ml of the suspension of *Sorangium cellulosum* Soce-90 BCE 33/10 from a liquid N<sub>2</sub> ampoule is transferred to 10 ml of G52 medium (in a 50 ml Erlenmeyer flask) and incubated for 3 days at 180 rpm in an agitator at 30°C, 25 mm displacement. 5 ml of this culture is added to 45 ml of G52 medium (in a 200 ml Erlenmeyer flask) and incubated for 3 days at 180 rpm in an agitator at 30°C, 25 mm displacement. 50 ml of this culture is then added to 450 ml of G52 medium (in a 2 litre Erlenmeyer flask) and incubated for 3 days at 180 rpm in an agitator at 30°C, 50 mm displacement.

Maintenance culture: The culture is overseeded every 3-4 days, by adding 50 ml of culture to 450 ml of G52 medium (in a 2 litre Erlenmeyer flask). All experiments and fermentations are carried out by starting with this maintenance culture.

Tests in a flask:

(i) Preculture in an agitating flask:

Starting with the 500 ml of maintenance culture, 1 x 450 ml of G52 medium are seeded with 50 ml of the maintenance culture and incubated for 4 days at 180 rpm in an agitator at 30°C, 50 mm displacement.

(ii) Main culture in the agitating flask:

40 ml of 1B12 medium plus 5 g/l 4-morpholine-propane-sulfonic acid (= MOPS) powder (in a 200 ml Erlenmeyer flask) are mixed with 5 ml of a 10x concentrated cyclodextrin solution, seeded with 10 ml of preculture and incubated for 5 days at 180 rpm in an agitator at 30°C, 50 mm displacement.

Fermentation: Fermentations are carried out on a scale of 10 litres, 100 litres and 500 litres. 20 litre and 100 litre fermentations serve as an intermediate culture step. Whereas the



precultures and intermediate cultures are seeded as the maintenance culture 10% (v/v), the main cultures are seeded with 20% (v/v) of the intermediate culture. Important: In contrast to the agitating cultures, the ingredients of the media for the fermentation are calculated on the final culture volume including the inoculum. If, for example, 18 litres of medium + 2 litres of inoculum are combined, then substances for 20 litres are weighed in, but are only mixed with 18 litres!

Preculture in an agitating flask:

Starting with the 500 ml maintenance culture, 4 x 450 ml of G52 medium (in a 2 litre Erlenmeyer flask) are each seeded with 50 ml thereof, and incubated for 4 days at 180 rpm in an agitator at 30°C, 50 mm displacement.

Intermediate culture, 20 litres or 100 litres:

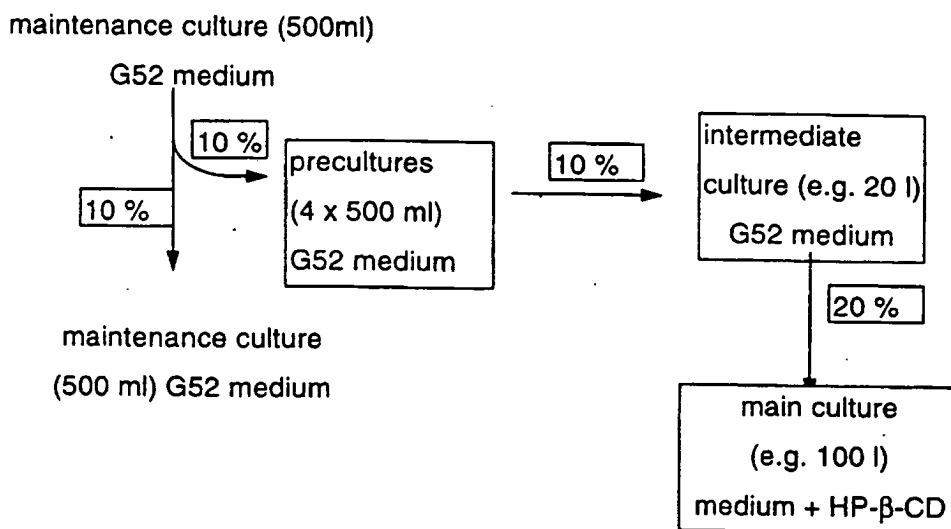
20 litres: 18 litres of G52 medium in a fermenter having a total volume of 30 litres are seeded with 2 litres of the preculture. Cultivation lasts for 3-4 days, and the conditions are: 30°C, 250 rpm, 0.5 litres of air per litre liquid per min, 0.5 bars excess pressure, no pH control.

100 litres: 90 litres of G52 medium in a fermenter having a total volume of 150 litres are seeded with 10 litres of the 20 litre intermediate culture. Cultivation lasts for 3-4 days, and the conditions are: 30°C, 150 rpm, 0.5 litres of air per litre liquid per min, 0.5 bars excess pressure, no pH control.

Main culture, 10 litres, 100 litres or 500 litres:

10 litres: The media substances for 10 litres of 1B12 medium are sterilised in 7 litres of water, then 1 litre of a sterile 10% 2-(hydroxypropyl) - $\beta$ -cyclodextrin solution are added, and seeded with 2 litres of a 20 litre intermediate culture. The duration of the main culture is 6-7 days, and the conditions are: 30°C, 250 rpm, 0.5 litres of air per litre of liquid per min, 0.5 bars excess pressure, pH control with H<sub>2</sub>SO<sub>4</sub>/KOH to pH 7.6 +/- 0.5 (i.e. no control between pH 7.1 and 8.1).

100 litres: The media substances for 100 litres of 1B12 medium are sterilised in 70 litres of water, then 10 litres of a sterile 10% 2-(hydroxypropyl) - $\beta$ -cyclodextrin solution are added, and seeded with 20 litres of a 20 litre intermediate culture. The duration of the main culture is 6-7 days, and the conditions are: 30°C, 200 rpm, 0.5 litres air per litre liquid per min., 0.5 bars excess pressure, pH control with H<sub>2</sub>SO<sub>4</sub>/KOH to pH 7.6 +/- 0.5. The chain of seeding for a 100 litre fermentation is shown schematically as follows:



500 litres: The media substances for 500 litres of 1B12 medium are sterilised in 350 litres of water, then 50 litres of a sterile 10% 2-(hydroxypropyl) - $\beta$ -cyclodextrin solution are added, and seeded with 100 litres of a 100 litre intermediate culture. The duration of the main culture is 6-7 days, and the conditions are: 30°C, 120 rpm, 0.5 litres air per litre liquid per min., 0.5 bars excess pressure, pH control with H<sub>2</sub>SO<sub>4</sub>/KOH to pH 7.6 +/- 0.5.

#### Product analysis:

#### Preparation of the sample:

50 ml samples are mixed with 2 ml of polystyrene resin Amberlite XAD16 (Rohm + Haas, Frankfurt, Germany) and shaken at 180 rpm for one hour at 30°C. The resin is subsequently filtered using a 150  $\mu$ m nylon sieve, washed with a little water and then added together with the filter to a 15 ml Nunc tube.

#### Elution of the product from the resin:

10 ml of isopropanol (>99%) are added to the tube with the filter and the resin. Afterwards, the sealed tube is shaken for 30 minutes at room temperature on a Rota-Mixer (Labinco BV, Netherlands). Then, 2 ml of the liquid are centrifuged off and the supernatant is added using a pipette to HPLC tubes.

HPLC analysis:

Column: Waters-Symetry C18, 100 x 4 mm, 3.5 µm  
 WAT066220 + preliminary column 3.9 x 20 mm  
 WAT054225

Solvents: A: 0.02 % phosphoric acid  
 B: Acetonitrile (HPLC-Quality)

Gradient: 41% B from 0 to 7 min.  
 100% B from 7.2 to 7.8 min.  
 41% B from 8 to 12 min.

Oven temp.: 30°C

Detection: 250 nm, UV-DAD detection

Injection vol.: 10 µl

Retention time: Epo A: 4.30 min      Epo B: 5.38 min

Example 2A: Effect of the addition of cyclodextrin and cyclodextrin derivatives to the epothilone concentrations attained.

All the cyclodextrin derivatives tested here come from the company Fluka, Buchs, CH. The tests are carried out in 200 ml agitating flasks with 50 ml culture volume. As controls, flasks with adsorber resin Amberlite XAD-16 (Rohm & Haas, Frankfurt, Germany) and without any adsorber addition are used. After incubation for 5 days, the following epothilone titres can be determined by HPLC:

Table 1:

| Addition                       | order<br>No. | Conc<br>[%w/v] <sup>1</sup> | Epo A [mg/l] | Epo B [mg/l] |
|--------------------------------|--------------|-----------------------------|--------------|--------------|
| Amberlite XAD-16 (v/v)         |              | 2.0 (%v/v)                  | 9.2          | 3.8          |
| 2-hydroxypropyl-β-cyclodextrin | 56332        | 0.1                         | 2.7          | 1.7          |
| 2-hydroxypropyl-β-cyclodextrin | "            | 0.5                         | 4.7          | 3.3          |

| Addition                                | order No. | Conc [%w/v] <sup>1</sup> | Epo A [mg/l] | Epo B [mg/l] |
|---|-----------|--------------------------|--------------|--------------|
| 2-hydroxypropyl- $\beta$ -cyclodextrin  | "         | 1.0                      | 4.7          | 3.4          |
| 2-hydroxypropyl- $\beta$ -cyclodextrin  | "         | 2.0                      | 4.7          | 4.1          |
| 2-hydroxypropyl- $\beta$ -cyclodextrin  | "         | 5.0                      | 1.7          | 0.5          |
| 2-hydroxypropyl- $\alpha$ -cyclodextrin | 56330     | 0.5                      | 1.2          | 1.2          |
| 2-hydroxypropyl- $\alpha$ -cyclodextrin | "         | 1.0                      | 1.2          | 1.2          |
| 2-hydroxypropyl- $\alpha$ -cyclodextrin | "         | 5.0                      | 2.5          | 2.3          |
| $\beta$ -cyclodextrin                   | 28707     | 0.1                      | 1.6          | 1.3          |
| $\beta$ -cyclodextrin                   | "         | 0.5                      | 3.6          | 2.5          |
| $\beta$ -cyclodextrin                   | "         | 1.0                      | 4.8          | 3.7          |
| $\beta$ -cyclodextrin                   | "         | 2.0                      | 4.8          | 2.9          |
| $\beta$ -cyclodextrin                   | "         | 5.0                      | 1.1          | 0.4          |
| methyl- $\beta$ -cyclodextrin           | 66292     | 0.5                      | 0.8          | <0.3         |
| methyl- $\beta$ -cyclodextrin           | "         | 1.0                      | <0.3         | <0.3         |
| methyl- $\beta$ -cyclodextrin           | "         | 2.0                      | <0.3         | <0.3         |
| 2,6 di-o-methyl- $\beta$ -cyclodextrin  | 39915     | 1.0                      | <0.3         | <0.3         |
| 2-hydroxypropyl- $\gamma$ -cyclodextrin | 56334     | 0.1                      | 0.3          | <0.3         |
| 2-hydroxypropyl- $\gamma$ -cyclodextrin | "         | 0.5                      | 0.9          | 0.8          |
| 2-hydroxypropyl- $\gamma$ -cyclodextrin | "         | 1.0                      | 1.1          | 0.7          |
| 2-hydroxypropyl- $\gamma$ -cyclodextrin | "         | 2.0                      | 2.6          | 0.7          |
| 2-hydroxypropyl- $\gamma$ -cyclodextrin | "         | 5.0                      | 5.0          | 1.1          |
| <b>no addition</b>                      |           |                          | <b>0.5</b>   | <b>0.5</b>   |

<sup>1</sup>) Apart from Amberlite (%v/v), all percentages are by weight (%w/v).

Few of the cyclodextrins tested (2,6-di-o-methyl- $\beta$ -cyclodextrin, methyl- $\beta$ -cyclodextrin) display no effect or a negative effect on epothilone production at the concentrations used. 1-2% 2-hydroxy-propyl- $\beta$ -cyclodextrin and  $\beta$ -cyclodextrin increase epothilone production in the examples by 6 to 8 times compared with production using no cyclodextrins.

**Example 2B: 10 litre fermentation with 1% 2-(hydroxypropyl)- $\beta$ -cyclodextrin:**

Fermentation is carried out in a 15 litre glass fermenter. The medium contains 10 g/l of 2-(hydroxypropyl)- $\beta$ -cyclodextrin from Wacker Chemie, Munich, Germany. The progress of fermentation is illustrated in Table 2. Fermentation is ended after 6 days and working up takes place.

Table 2: Progress of a 10 litre fermentation

| duration of culture [d] | Epothilone A [mg/l] | Epothilone B [mg/l] |
|-------------------------|---------------------|---------------------|
| 0                       | 0                   | 0                   |
| 1                       | 0                   | 0                   |
| 2                       | 0.5                 | 0.3                 |
| 3                       | 1.8                 | 2.5                 |
| 4                       | 3.0                 | 5.1                 |
| 5                       | 3.7                 | 5.9                 |
| 6                       | 3.6                 | 5.7                 |

**Example 2C: 100 litre fermentation with 1% 2-(hydroxypropyl)- $\beta$ -cyclodextrin:**

Fermentation is carried out in a 150 litre fermenter. The medium contains 10 g/l of 2-(Hydroxypropyl)- $\beta$ -cyclodextrin. The progress of fermentation is illustrated in Table 3. The fermentation is harvested after 7 days and worked up.

Table 3: Progress of a 100 litre fermentation

| duration of culture [d] | Epothilone A [mg/l] | Epothilone B [mg/l] |
|-------------------------|---------------------|---------------------|
| 0                       | 0                   | 0                   |

|   |     |     |
|---|-----|-----|
| 1 | 0   | 0   |
| 2 | 0.3 | 0   |
| 3 | 0.9 | 1.1 |
| 4 | 1.5 | 2.3 |
| 5 | 1.6 | 3.3 |
| 6 | 1.8 | 3.7 |
| 7 | 1.8 | 3.5 |

**Example 2D: 500 litre fermentation with 1% 2-(hydroxypropyl)- $\beta$ -cyclodextrin:**

Fermentation is carried out in a 750 litre fermenter. The medium contains 10 g/l of 2-(Hydroxypropyl)- $\beta$ -cyclodextrin. The progress of fermentation is illustrated in Table 4. The fermentation is harvested after 7 days and worked up.

**Table 4: Progress of a 500 litre fermentation**

| duration of culture [d] | Epothilone A [mg/l] | Epothilone B [mg/l] |
|-------------------------|---------------------|---------------------|
| 0                       | 0                   | 0                   |
| 1                       | 0                   | 0                   |
| 2                       | 0                   | 0                   |
| 3                       | 0.6                 | 0.6                 |
| 4                       | 1.7                 | 2.2                 |
| 5                       | 3.1                 | 4.5                 |
| 6                       | 3.1                 | 5.1                 |

**Example 2E: Comparison example 10 litre fermentation without adding an adsorber:**

Fermentation is carried out in a 15 litre glass fermenter. The medium does not contain any cyclodextrin or other adsorber. The progress of fermentation is illustrated in Table 5. The fermentation is not harvested and worked up.

Table 5: Progress of a 10 litre fermentation without adsorber.

| duration of culture [d] | Epothilone A<br>[mg/l] | Epothilone B<br>[mg/l] |
|-------------------------|------------------------|------------------------|
| 0                       | 0                      | 0                      |
| 1                       | 0                      | 0                      |
| 2                       | 0                      | 0                      |
| 3                       | 0                      | 0                      |
| 4                       | 0.7                    | 0.7                    |
| 5                       | 0.7                    | 1.0                    |
| 6                       | 0.8                    | 1.3                    |

Example 3: Working up of the epothilones: Isolation from a 500 litre main culture:

The volume of harvest from the 500 litre main culture of example 2D is 450 litres and is separated using a Westfalia clarifying separator Type SA-20-06 (rpm = 6500) into the liquid phase (centrifugate + rinsing water = 650 litres) and solid phase (cells = ca. 15 kg). The main part of the epothilones are found in the centrifugate. The centrifuged cell pulp contains < 15% of the determined epothilone portion and is not further processed. The 650 litre centrifugate is then placed in a 4000 litre stirring vessel, mixed with 10 litres of Amberlite XAD-16 (centrifugate:resin volume = 65:1) and stirred. After a period of contact of ca. 2 hours, the resin is centrifuged away in a Heine overflow centrifuge (basket content 40 litres; rpm = 2800). The resin is discharged from the centrifuge and washed with 10-15 litres of deionised water. Desorption is effected by stirring the resin twice, each time in portions with 30 litres of isopropanol in 30 litre glass stirring vessels for 30 minutes. Separation of the isopropanol phase from the resin takes place using a suction filter. The isopropanol is then removed from the combined isopropanol phases by adding 15-20 litres of water in a vacuum-operated circulating evaporator (Schmid-Verdampfer) and the resulting water

phase of ca. 10 litres is extracted 3x each time with 10 litres of ethyl acetate. Extraction is effected in 30 litre glass stirring vessels. The ethyl acetate extract is concentrated to 3-5 litres in a vacuum-operated circulating evaporator (Schmid-Verdampfer) and afterwards concentrated to dryness in a rotary evaporator (Büchi type) under vacuum. The result is an ethyl acetate extract of 50.2 g. The ethyl acetate extract is dissolved in 500 ml of methanol, the insoluble portions filtered off using a folded filter, and the solution added to a 10 kg Sephadex LH 20 column (Pharmacia, Uppsala, Sweden) (column diameter 20 cm, filling level ca. 1.2 m). Elution is effected with methanol as eluant. Epothilone A and B is present predominantly in fractions 21-23 (at a fraction size of 1 litre). These fractions are concentrated to dryness in a vacuum on a rotary evaporator (total weight 9.0 g). These Sephadex peak fractions (9.0 g) are thereafter dissolved in 92 ml of acetonitrile:water:methylene chloride = 50:40:2, the solution filtered through a folded filter and added to a RP column (equipment Prepbar 200, Merck; 2.0 kg LiChrospher RP-18 Merck, grain size 12µm, column diameter 10 cm, filling level 42 cm; Merck, Darmstadt, Germany). Elution is effected with acetonitrile:water = 3:7 (flow rate = 500 ml/min.; retention time of epothilone A = ca. 51-59 mins.; retention time of epothilone B = ca. 60-69 mins.). Fractionation is monitored with a UV detector at 250 nm. The fractions are concentrated to dryness under vacuum on a Büchi-Rotavapor rotary evaporator. The weight of the epothilone A peak fraction is 700 mg, and according to HPLC (external standard) it has a content of 75.1%. That of the epothilone B peak fraction is 1980 mg, and the content according to HPLC (external standard) is 86.6%. Finally, the epothilone A fraction (700 mg) is crystallised from 5 ml of ethyl acetate:toluene = 2:3, and yields 170 mg of epothilone A pure crystallisate [content according to HPLC (% of area) = 94.3%]. Crystallisation of the epothilone B fraction (1980 mg) is effected from 18 ml of methanol and yields 1440 mg of epothilone B pure crystallisate [content according to HPLC (% of area) = 99.2%]. m.p. (Epothilone B): e.g. 124-125 °C; <sup>1</sup>H-NMR data for Epothilone B: 500 MHz-NMR, solvent: DMSO-d<sub>6</sub>. Chemical displacement δ in ppm relative to TMS. s = singlet; d = doublet; m = multiplet

δ (Multiplicity)

Integral (number of H)

7.34 (s)

1



|                           |   |
|---------------------------|---|
| 6.50 (s)                  | 1 |
| 5.28 (d)                  | 1 |
| 5.08 (d)                  | 1 |
| 4.46 (d)                  | 1 |
| 4.08 (m)                  | 1 |
| 3.47 (m)                  | 1 |
| 3.11 (m)                  | 1 |
| 2.83 (dd)                 | 1 |
| 2.64 (s)                  | 3 |
| 2.36 (m)                  | 2 |
| 2.09 (s)                  | 3 |
| 2.04 (m)                  | 1 |
| 1.83 (m)                  | 1 |
| 1.61 (m)                  | 1 |
| 1.47 - 1.24 (m)           | 4 |
| 1.18 (s)                  | 6 |
| 1.13 (m)                  | 2 |
| 1.06 (d)                  | 3 |
| 0.89 (d + s, overlapping) | 6 |

 $\Sigma = 41$

In this example (Example 3), epothilone B is obtained in the crystal modification A, which is characterised by the X-ray diffraction diagram of modification A (see general part of the present disclosure).

Example 4: Crystal modification B of Epothilone B

50 mg of epothilone B (obtained for example as above) are suspended in 1 ml of isopropanol and shaken for 24 hours at 25 °C. The product is filtered and dried. After drying under a high vacuum, epothilones B are obtained in the form of white crystals. The crystal modification of the product is characterised by the X-ray diffraction diagram of modification B (see general part of the present disclosure).

BUDAPEST TREATY ON THE INTERNATIONAL  
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS  
FOR THE PURPOSES OF PATENT PROCEDURE

## INTERNATIONAL FORM

Novartis Pharma AG  
Postfach  
CH-4002 Basel

## VIABILITY STATEMENT

issued pursuant to Rule 10.2 by the  
INTERNATIONAL DEPOSITARY AUTHORITY  
identified at the bottom of this page

|  |  |
|--|--|
| I. DEPOSITOR   | II. IDENTIFICATION OF THE MICROORGANISM  |
| Name: Novartis Pharma AG<br>Postfach<br>Address: CH-4002 Basel   | Accession number given by the<br>INTERNATIONAL DEPOSITARY AUTHORITY:<br>DSM 11999<br><br>Date of the deposit or the transfer <sup>1</sup> :<br>1998-02-09                      |
| III. VIABILITY STATEMENT   |  |
| The viability of the microorganism identified under II above was tested on 1998-02-09 <sup>2</sup> .<br>On that date, the said microorganism was<br><br>(X) <sup>3</sup> viable<br><br>( ) <sup>3</sup> no longer viable |  |
| IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED <sup>4</sup>  |  |
|  |  |
| V. INTERNATIONAL DEPOSITARY AUTHORITY  |  |
| Name: DSMZ-DEUTSCHE SAMMLUNG VON<br>MIKROORGANISMEN UND ZELLKULTUREN GmbH<br><br>Address: Mascheroder Weg 1b<br>D-38124 Braunschweig   | Signature(s) of person(s) having the power to represent the<br>International Depositary Authority or of authorized official(s):<br><br><i>U. W. L.</i><br><br>Date: 1998-02-19 |

- <sup>1</sup> Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
- <sup>2</sup> In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.
- <sup>3</sup> Mark with a cross the applicable box.
- <sup>4</sup> Fill in if the information has been requested and if the results of the test were negative.

BUDAPEST TREATY ON THE INTERNATIONAL  
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS  
FOR THE PURPOSES OF PATENT PROCEDURE

## INTERNATIONAL FORM

Novartis Pharma AG  
Postfach  
CH-4002 BaselRECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT  
issued pursuant to Rule 7.1 by the  
INTERNATIONAL DEPOSITARY AUTHORITY  
identified at the bottom of this page

|  |   |
|--|---|
| I. IDENTIFICATION OF THE MICROORGANISM   |   |
| Identification reference given by the DEPOSITOR:<br>BCE33/10   | Accession number given by the<br>INTERNATIONAL DEPOSITARY AUTHORITY:<br><br>DSM 11999   |
| II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION   |   |
| The microorganism identified under I. above was accompanied by:<br><br><input type="checkbox"/> a scientific description<br><input checked="" type="checkbox"/> a proposed taxonomic designation<br><br>(Mark with a cross where applicable).  |   |
| III. RECEIPT AND ACCEPTANCE  |   |
| This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 1998-02-09<br>(Date of the original deposit).   |   |
| IV. RECEIPT OF REQUEST FOR CONVERSION  |   |
| The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit)<br>and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request<br>for conversion). |   |
| V. INTERNATIONAL DEPOSITARY AUTHORITY  |   |
| Name: DSMZ-DEUTSCHE SAMMLUNG VON<br>MIKROORGANISMEN UND ZELLKULTUREN GmbH<br><br>Address: Mascheroder Weg 1b<br>D-38124 Braunschweig   | Signature(s) of person(s) having the power to represent the<br>International Depositary Authority or of authorized official(s):<br><br><i>V. Waichs</i><br><br>Date: 1998-02-19 |

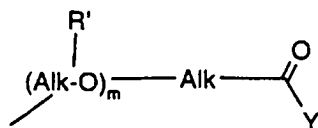
<sup>1</sup> Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

What is claimed is:

1. A process for concentrating epothilones in a culture medium for the biotechnological preparation of these compounds, the process comprising microorganisms which produce these compounds, whereby a complex-forming component which is soluble in the culture medium is added to the medium.
2. A process according to claim 1 for concentrating epothilones in a culture medium for the biotechnological preparation of these compounds, the process comprising myxobacteria as producers of natural substances, whereby a complex-forming component which is soluble in the culture medium is added to the medium.
3. A process according to claim 1 for concentrating epothilones, in which a culture medium is used for the biotechnological preparation of epothilones, the medium containing a *Sorangium* strain suitable for the preparation thereof, water and other suitable customary components of culture media,  
wherein one or more cyclodextrins are added to the medium, the cyclodextrins being selected from  $\alpha$ -cyclodextrin,  $\beta$ -cyclodextrin,  $\gamma$ -cyclodextrin,  $\delta$ -cyclodextrin,  $\epsilon$ -cyclodextrin,  $\zeta$ -cyclodextrin,  $\eta$ -cyclodextrin and  $\theta$ -cyclodextrin; or a cyclodextrin derivative or a mixture of cyclodextrin derivatives selected from derivatives of a cyclodextrin in which one or more up to all of the hydroxy groups are etherified to an alkyl ether, an aryl-hydroxyalkyl ether; a hydroxyalkyl ether; a carboxyalkyl ether; a derivatised carboxy lower alkyl ether in which the derivatised carboxy is aminocarbonyl, mono- or di-lower-alkylaminocarbonyl, morpholino-, piperidino-, pyrrolidino- or piperazino-carbonyl, or alkyloxycarbonyl; a sulfoalkyl ether; a cyclodextrin in which one or more OH groups are etherified with a radical of formula



wherein alk is alkyl and n is a whole number from and including 2 up to and including 12; a cyclodextrin in which one or more OH groups are etherified with a radical of formula



wherein R' is hydrogen, hydroxy, -O-(alk-O)<sub>z</sub>-H, -O-(alk(-R)-O)<sub>p</sub>-H or -O-(alk(-R)-O)<sub>q</sub>-alk-CO-Y; alk in all cases is alkyl; m, n, p, q and z are a whole number from 1 to 12; and Y is OR<sub>1</sub> or NR<sub>2</sub>R<sub>3</sub>, wherein R<sub>1</sub>, R<sub>2</sub> and R<sub>3</sub>, independently of one another, are hydrogen or lower alkyl, or R<sub>2</sub> and R<sub>3</sub>, combined with the binding nitrogen signify morpholino, piperidino, pyrrolidino or piperazino; or a branched cyclodextrin in which etherifications or acetals exist with other sugar molecules, and which are selected from glucosyl-, diglucosyl- (G<sub>2</sub>-β-cyclodextrin), maltosyl- and dimaltosyl-cyclodextrin, or N-acetylglucosaminyl-, glucosaminyl-, N-acetylgalactosaminyl- and galactosaminyl-cyclodextrin; and a lower alkanoyl-, such as acetyler of a cyclodextrin; or mixtures of two or more of the said cyclodextrin and/or cyclodextrin derivatives.

4. Process according to claim 3, in which the cyclodextrin and/or the cyclodextrin derivative is added to the culture medium in a concentration of between 0.05 and 10 percent by weight (w/v).
5. Process according to claim 4, in which the cyclodextrin and/or the cyclodextrin derivative is added in a concentration of between 0.1 and 2 percent by weight.
6. A process according to claim 3, in which the cyclodextrin derivative is selected from a cyclodextrin and a hydroxy lower alkyl cyclodextrin; or mixtures of one or more thereof.
7. A process according to claim 1, in which the complex-forming component is 2-hydroxy-propyl-β-cyclodextrin.
8. A culture medium which comprises a complex-forming component and a microorganism suitable for the production of epothilones.
9. A culture medium according to claim 8, in which the complex-forming component used is a cyclodextrin, a cyclodextrin derivative or a mixture of two or more complex-forming components selected from cyclodextrins and cyclodextrin derivatives.

10. A culture medium according to claim 9, in which the microorganism is a myxobacterium.

11. A process for the production of epothilones, in which the epothilones are obtained by working up a culture medium for the biotechnological preparation of these compounds, the medium comprising microorganisms which are suitable for producing epothilones, especially myxobacteria, as producers of natural substances, to which medium is added a complex-forming component which is soluble in the culture medium, and subsequently purifying and, if desired, separating the epothilones.

12. A process according to claim 11, wherein the epothilones are obtained by working up a culture medium for the biotechnological preparation of these compounds, the culture medium containing a myxobacterium of the genus *Sorangium*, and to which medium is added a complex-forming component which is soluble in the culture medium, the culture is separated into the solid and the liquid phase (centrifugate) by centrifugation; the centrifugate is mixed with a resin or is run over a column filled with such a resin; if necessary the resin is washed with water; the epothilone(s) is or are desorbed from the resin with a polar solvent; if necessary this is concentrated with prior, simultaneous or subsequent addition of water; an organic solvent which is immiscible with water is added and the epothilone(s) is or are transferred into the organic phase; the organic phase obtained is concentrated if required; the epothilones from the organic solution obtained are concentrated through a molecular sieve for compounds of low molecular weight; and subsequently the fractions containing the epothilones undergo separation on a reversed-phase column; whereby epothilones A and B are obtained separately and, if desired, can be further concentrated by recrystallisation.

13. A process for the production of epothilones, which

- a) is a process for concentrating epothilones in a culture medium for the biotechnological preparation of these compounds, which contains a microorganism suitable for the preparation thereof, water and other suitable customary constituents of culture media, whereby a cyclodextrin or a cyclodextrin derivative is added to the medium, or a mixture of two or more of these compounds; and
- b) comprises a step for separating epothilones from one another, which is characterised by chromatography on a reversed-phase column with an eluant containing a lower alkyl-

cyanide, whereby chromatography is carried out on column material charged with hydrocarbon chains, and an eluant containing a lower alkylnitrile is used; whereby if desired further working up steps and purification steps are possible.

14. A method of separating epothilones from one another, especially epothilones A and B, which is characterised by chromatography on a reversed-phase column with an eluant containing a lower alkylcyanide.

15. A method according to claim 14, wherein a column material is used, which is charged with hydrocarbon chains containing 18 carbon atoms and the eluant used is a mixture of water and acetonitrile.

16. A strain of *Sorangium cellulosum*, obtained by mutagenesis, which under otherwise identical conditions, produces more epothilones than *Sorangium cellulosum* Soce90.

17. A strain according to claim 16, having the reference BCE33/10.

18. A crystal form of epothilone B having the reference modification A, which is characterised by the X-ray diffraction diagram reproduced in the form of a table, obtained using a diffractometer with Cu-K $\alpha_1$  radiation.

| 2 $\theta$ | Intensity   |
|------------|-------------|
| 7.7        | very strong |
| 10.6       | weak        |
| 13.6       | average     |
| 14.4       | average     |
| 15.5       | average     |
| 16.4       | weak        |
| 16.8       | weak        |
| 17.1       | weak        |
| 17.3       | weak        |
| 17.7       | weak        |
| 18.5       | weak        |
| 20.7       | strong      |



|      |         |
|------|---------|
| 21.2 | strong  |
| 21.9 | weak    |
| 22.4 | weak    |
| 23.3 | strong  |
| 25.9 | average |
| 31.2 | weak    |
| 32.0 | average |

19. A crystal form of epothilone B having the reference modification B, which is characterised by the X-ray diffraction diagram reproduced in the form of a table, obtained using a diffractometer with Cu-K $\alpha_1$  radiation.

|            |             |
|------------|-------------|
| 2 $\theta$ | Intensity   |
| 6.9        | very strong |
| 8.0        | weak        |
| 8.3        | average     |
| 10.8       | strong      |
| 11.5       | average     |
| 12.4       | weak        |
| 13.1       | strong      |
| 15.5       | weak        |
| 16.2       | weak        |
| 16.7       | average     |
| 18.1       | average     |
| 18.6       | average     |
| 20.4       | weak        |
| 20.9       | strong      |
| 21.3       | weak        |
| 21.5       | very weak   |
| 22.5       | average     |
| 24.2       | weak        |
| 25.1       | average     |

20. A pharmaceutical composition which contains an active ingredient according to one of claims 18 and 19, together with a pharmaceutically acceptable carrier.

21. Method of treating a warm-blooded animal suffering from a proliferative disease, by administering a dosage of epothilone B which is effective for treating said disease, according to one of claims 18 and 19, to a warm-blooded animal requiring such treatment.

22. Use of a crystal form according to one of claims 18 and 19 in the treatment of a proliferative disease.

23. Use of a new crystal form of epothilone B according to one of claims 18 and 19 in the production of pharmaceutical preparations, whereby a crystal form of this type is mixed with one or more carriers.

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

|  |  |   |
|--|--|---|
| <b>(51) International Patent Classification <sup>6</sup> :</b><br><b>C12P 17/18, C07D 493/04, B01D</b><br><b>15/08 // (C12P 17/18, C12R 1:01)</b>  | <b>A3</b>  | <b>(11) International Publication Number:</b> <b>WO 99/42602</b><br><b>(43) International Publication Date:</b> 26 August 1999 (26.08.99) |
| <b>(21) International Application Number:</b> PCT/EP99/01025<br><b>(22) International Filing Date:</b> 17 February 1999 (17.02.99)<br><br><b>(30) Priority Data:</b><br>396/98                      19 February 1998 (19.02.98)      CH<br>1007/98                     5 May 1998 (05.05.98)                      CH<br><br><b>(71) Applicant (for all designated States except AT):</b> NOVARTIS AG [CH/CH]; Schwarzwaldallee 215, D-4058 Basel (CH).<br><b>(71) Applicant (for AT only):</b> NOVARTIS-ERFINDUNGEN VERWALTUNGSGESELLSCHAFT MBH [AT/AT]; Brunner Strasse 59, A-1235 Vienna (AT).<br><br><b>(72) Inventors:</b> HOFMANN, Hans; Bottmingerstrasse 31, CH-4107 Ettingen (CH). MAHNKE, Marion; Birkenweg 1, D-79585 Steinen (DE). MEMMERT, Klaus; Erlenweg 11, D-79540 Lörrach (DE). PETERSEN, Frank; Eimeldingerweg 8, D-79576 Weil am Rhein (DE). SCHUPP, Thomas; Fröschmattweg 5, CH-4313 Möhlin (CH). KÜSTERS, Ernst; Gallenweilerweg 1, D-79427 Eschbach (DE). MUTZ, Michael; Schlüsselstrasse 30, D-79104 Freiburg (DE). | <b>(74) Agent:</b> BECKER, Konrad; Novartis AG, Corporate Intellectual Property, Patent & Trademark Dept., CH-4002 Basel (CH).<br><br><b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).<br><br><b>Published</b><br><i>With international search report.</i><br><i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i><br><br><b>(88) Date of publication of the international search report:</b><br>25 November 1999 (25.11.99) |   |
| <b>(54) Title:</b> FERMENTATIVE PREPARATION PROCESS FOR CYTOSTATICS AND CRYSTAL FORMS THEREOF<br><br><b>(57) Abstract</b><br><br>The invention relates to a process for concentrating epothilones in culture media, a process for the production of epothilones, a process for separating epothilones A and B and a strain obtained by mutagenesis for the production of epothilones, as well as aspects related thereto. Crystal forms of epothilone B are also described.  |  |   |

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Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

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| DK | Denmark                  | LR | Liberia                                  | SG | Singapore                                    |    |                          |
| EE | Estonia                  |    |  |    |  |    |                          |

# INTERNATIONAL SEARCH REPORT

Internat I Application No  
PCT/EP 99/01025

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12P17/18 C07D493/04 B01D15/08  
C12R1:01)

//(C12P17/18,

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12P C07D B01D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No. |
|------------|---|-----------------------|
| A          | WO 93 10121 A (GESELLSCHAFT FÜR BIOTECHNOLOGISCHE FORSCHUNG MBH)<br>27 May 1993 (1993-05-27)<br>cited in the application<br>page 7, paragraph 4 -page 9, paragraph 2;<br>claim 5<br>--- | 1-13                  |
| A          | DE 42 07 922 A (PHARMATECH GMBH)<br>23 September 1993 (1993-09-23)<br>page 2, line 47 -page 3, line 26<br>---   | 1-13                  |
| A          | WO 94 26728 A (CHINOIN LTD.)<br>24 November 1994 (1994-11-24)<br>page 2, line 34 -page 3, line 28; examples<br>-----  | 1-13                  |

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
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- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
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Date of the actual completion of the international search

23 June 1999

Date of mailing of the international search report

08. 10. 99

Name and mailing address of the ISA

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MONTERO LOPEZ B.

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP 99/ 01025

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
  
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
  
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-13

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1-13

Procee for concentrating epothilones in a culture medium by addition of a complex-forminh component; culture medium for the production of epothilones comprising a complex-forming component and use thereof for the production of epothilones.

2. Claims: 14, 15

Method for separating epothilones from one another by chromatography on a reversed-phase column with an eluant containing a lower alkylcyanide.

3. Claims: 16, 17

Mutant strain of Sorangium cellulosum which produces more epothilones than Sorangium cellulosum Soce90.

4. Claims: 18-23

Crystal forms of epothilone B, pharmaceutical composition comprising the same and us thereof in the treatment of a proliferative disease

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Internat I Application No

PCT/EP 99/01025

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
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